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**Brn-3b and PPAR $\gamma$  nuclear receptor expression in adipose tissue of human  
subjects**

A Thesis in

Department of Health Science, Biomedical Sciences

By

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**Abstract:**

Obesity rates have reached alarming heights globally, having more than doubled since the 1980s. Over 60% of diabetic patients are obese, and the increasing severity of the disease is associated with Type 2 Diabetes Mellitus (T2DM) incidence, which is reversible by weight loss. Understanding the molecular mechanisms linking obesity and T2DM could trigger the development of new medication, procedures and perhaps devices.

Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) is a nuclear receptor expressed in adipose tissue and regulates the expression of genes involved in both adipocyte differentiation and lipid metabolism. Another novel transcription factor, Brn-3b, known to be involved in neuronal development, has recently been shown to be expressed in murine adipose tissue and also in peripheral blood cells (PBCs) obtained from morbidly obese patients. Furthermore, the expression of Brn-3b was significantly reduced in PBCs of diabetic patients.

The aim of this study was to investigate the expression of PPAR $\gamma$  and Brn-3b in cells of the adipose tissue of obese patients undergoing weight-reducing sleeve gastrectomy. Anthropometric and biochemical data were collected for all patients. Samples from two abdominal adipose tissue depots (subcutaneous and omental) were also obtained from each patient. RNA was extracted and qPCR for PPAR $\gamma$  and Brn-3b was performed.

Our results showed that PPAR $\gamma$  is highly expressed in the adipocytes of omental adipose tissue when compared to the adipocytes of the subcutaneous adipose tissue, while Brn-3b was not detectable in adipocytes, but exclusively expressed in the stromal vascular fraction, especially of the subcutaneous adipose tissue. The expression of Brn-3b increased with rising levels of systemic insulin and was also elevated in the SVF of patients with metabolic disease compared with those without.

In conclusion our study indicated that there was a reciprocal relationship between the expression of PPAR $\gamma$  and Brn-3b that needs further investigation, and raises questions concerning the implication of Brn-3b in the pathology of obesity and T2DM.

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## List of abbreviations:

ADIPO	Adipocyte
Brn-3b	Brain-3b
BMI	Body Mass Index
DBD	DNA Binding Domain
GLUT2	Glucose Transporter 2
GLUT4	Glucose Transporter 4
HbA1c	Hemoglobin A1c
HDL	High Density Lipoprotein
IR	Insulin Resistance
LBD	Ligand Binding Domain
MHO	Metabolically Healthy Obese
mRNA	Messenger Ribonucleic acid
NTD	N-Terminal Domain
NR	Nuclear Receptor
NR1C1	Nuclear Receptor subfamily 1, group C, member 1
NR1C2	Nuclear Receptor subfamily 1, group C, member 2
NR1C3	Nuclear Receptor subfamily 1, group C, member 3
OM	Omental
PBMCs	Peripheral Blood Mononuclear Cells
PPRE	Peroxisome Proliferator Response Element
PPAR $\alpha$	Peroxisome Proliferator-Activated Receptor Alfa
PPAR $\beta$	Peroxisome Proliferator-Activated Receptor Beta



PPAR $\gamma$	Peroxisome Proliferator-Activated Receptor Gamma
PI3K	Phosphatidylinositide 3-kinases
PEPCK	Phosphoenolpyruvate carboxy kinase
PO	Pathologically Obese
POMC	Proopiomelanocortin
POU	Pituitary –Octamer –Unc-86 transcription factors
q-PCR	Quantitative Polymerase Chain Reaction
RXR	Retinoid X Receptor
RNA	Ribonucleic acid
SD	Standard Deviation
SVF	Stromal Vascular Fraction
SC	Subcutaneous
T2DM	Type 2 Diabetes Mellitus
TZDs	Thiazolidinedione's
TNF- $\alpha$	Tumor Necrosis Factor Alfa
WHO	World Health Organization

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# **CHAPTER 1: INTRODUCTION**

Obesity rates have reached alarming rates globally, having more than doubled since the 1980s. Over 60% of diabetic patients are obese, while increasing severity of obesity is associated with disproportionate increase in T2DM incidence and hence, weight loss is necessitated. Understanding the molecular mechanisms linking obesity and T2DM could trigger the development of new procedures, devices, and medication both for obese and non-obese patients.

Transcription factors are involved in the regulation of expression of multiple genes by binding to specific DNA sites of eukaryotic gene promoters. Given their roles as modulators of the expression of multiple target genes, they may be of utmost importance in the pathways involved in the development of obesity, insulin resistance (IR) and T2DM.

From the diverse pool of transcription factors, peroxisome proliferator-activator receptor- $\gamma$  (PPAR $\gamma$ ) has emerged as potentially important for the pathogenesis of obesity and IR. The PPAR $\gamma$  has been implicated in the regulation of lipid and glucose metabolism as well as cellular differentiation, especially adipogenesis. PPAR $\gamma$  is expressed in abundance in adipose tissue but it is also found in smaller quantities in skeletal muscle and other human tissues. It acts as a receptor for endogenous, e.g. fatty acids and prostaglandins, as well as exogenous ligands, including thiazolidinediones, a family of pharmacological agents which are insulin-sensitizing leading to improved glucose control, reduced inflammation and increased adipocyte differentiation. In humans, one study indicated that, compared to normal-weight controls, obese subjects who experienced significant weight gain before the study exhibited increased PPAR $\gamma$  mRNA levels in PBMC, while no difference was observed in other obese groups. As far as expression in whole blood is concerned, one study of limited scale has shown that in morbidly obese patients, PPAR $\gamma$  expression decreased with increasing Body Mass Index BMI. Investigating PPAR $\gamma$  expression in

whole blood appears intriguing because it is easy to obtain and contains peripheral immune cells, which are implicated in the pathogenesis of IR.

POU4F2/Brn-3b (referred to as Brn-3b) belongs to the POU (Pit-Oct-Unc) family of nuclear transcription factors. Importantly, Brn-3b has been detected in human PBMC. In contrast to the abundance of studies on PPAR- $\gamma$ , the study of Brn-3b in metabolic diseases is novel. A study in morbidly obese patients indicated that Brn-3b expression was significantly reduced in whole blood of diabetic versus non-diabetic subjects. These findings give rise to questions concerning the implication of Brn-3b in the pathology of obesity and T2DM.

Given the limited amount of data on these two transcription factors in humans, the study of differences in their mRNA expression between obese patients, with or without T2DM could provide useful information for the etiology of obesity and related co-morbidities.

In this study we aim to investigate the expression of PPAR $\gamma$  and Brn-3b in relation to obesity and surgery-induced weight loss.

## **CHAPTER 2: REVIEW OF THE LITERATURE**

**Obesity:**

World health Organization defined obesity as the “abnormal or excessive fat accumulation that may impair health”<sup>1</sup>. Clinically, obesity is measured by the Body Mass Index (BMI) of 30 Kg/m<sup>2</sup> or greater. BMI is a simple and costless tool that helps in measuring underweight, overweight and obesity. It is defined as weight in Kilogram divided by the height in meters squared. According to BMI, obesity can be classified into 3 classes: class I (BMI: 30- 34.9), class II (BMI: 35- 39.9), and class III (BMI: >40)<sup>2</sup>.

**Prevalence of obesity:**

The prevalence of obesity has doubled since 1980. The latest statistics from the WHO estimate that 11% of adults are obese worldwide<sup>1</sup>. Qatar is ranked sixth in the Middle East for its rate of obesity<sup>3</sup>. There is an increase in the mean BMI trends of males from 25.0 to 28.3 and females from 27.0 to 29.0 through the years 1980 to 2009<sup>3</sup>. A most recent study estimated the prevalence of obesity in Qatar is 40-50%, with greater prevalence in females compared to males (54.7% and 44% respectively)<sup>4</sup>.

**Causes of obesity:**

Obesity is a multifactorial disease, caused by the interaction of several environmental, genetics and human behavioral factors. Environmental factors are the main cause of obesity when there is excess energy intake compared to low physical activity. Studies support the effect of excess energy intake and physical inactivates on obesity. A study from England shows that the trend of obesity has increased in parallel with owning a car and watching television<sup>5</sup>. Other environmental factors that show increase in the prevalence of obesity are the quality of local parks where it is contribute to physical inactivity<sup>6</sup>.

Several studies have demonstrated the association of multiple gene loci with obesity. A linkage study done by Hixson and colleague, among Mexican Americans, identified a significant association between *POMC* (Proopiomelanocortin) locus in chromosome 2 and variations in the serum leptin levels<sup>7</sup>. Other linkage studies conducted on Caucasian

populations showed the association of obesity with genes on chromosome 3 (e.g. adiponectin, *GLUT2* and *PI3K*) and chromosome 17 (e.g. *GLUT4* and *PPARα*)<sup>8</sup>.

**Adipose tissue:**

Adipose tissue can be classified according to its anatomical distribution in the body to subcutaneous and omental fats. Anatomically subcutaneous adipose tissue is located in the mammary and femerogluteal regions, back and abdominal wall. Omental adipose tissue is located within the abdominal cavity between the organs. The omental depot contain have more inflammatory cells compared to subcutaneous fats<sup>9,10</sup>. Normally, fat is accumulated in subcutaneous adipose tissue with limited capacity in which if the capacity of fat exceeded or impaired, fat will accumulate in areas other than subcutaneous<sup>11</sup>. Fats are abnormally accumulated in omental adipose tissue as results of chronic stress and elevated cortisol level<sup>12</sup>. In a normal lean person, 80% of all body fat is subcutaneous while only 10-20% is located in omental area<sup>13</sup>.

Adipose tissue composed of adipocytes and stromal vascular fraction (SVF), which contain preadipocytes, fibroblasts, vascular cells, neural cells, and immune cells such as adipose tissue macrophages. Adipocytes are the main cellular component of adipose tissue and are the central store of energy in the form of triglycerides<sup>11</sup>. When adipocytes continue to grow they become larger in size and dysfunctional. These hypertrophied adipocytes are insulin resistant and hyperlipolytic. Overall the subcutaneous adipose tissue consists of larger adipocytes than those in the omental depot. Paradoxically, the smaller adipocytes of the subcutaneous depot are more metabolically active, insulin sensitive and have high avidity to uptake free fatty acids and triglycerides<sup>14,15</sup>. The adipocytes of the omental depot, despite being smaller, are more insulin resistant and inflammatory.

Accumulation of triglycerides in omental adipose tissue is associated with high risk for several diseases like hyperglycemia, hyperinsulinemia, and diabetes<sup>16,17</sup>. In addition to the

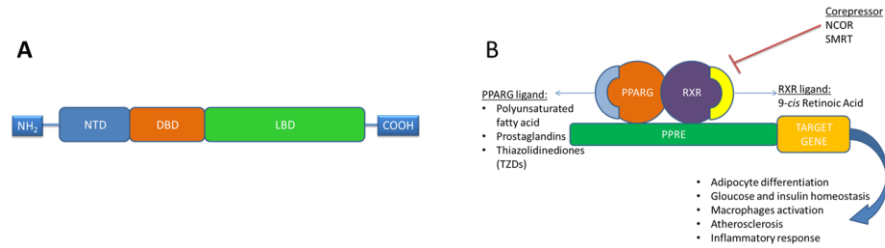


risk of metabolic syndromes, fat accumulation in the omental area is associated with the risk of developing cardiovascular disease and hypertension<sup>10, 18, 19</sup>.

### **Transcription factors and its role in obesity:**

#### **PPAR $\gamma$ :**

Peroxisome proliferator-activated receptors (PPARs) members of the nuclear receptor (NR) superfamily of ligand activated transcription factors<sup>20</sup>. There are three isotypes of PPARs: PPAR $\alpha$  (NR1C1), PPAR $\beta$  (NR1C2) and PPAR $\gamma$  (NR1C3)<sup>21,22</sup>. Although they are encoded by different genes, they share the same molecular structures as most NRs<sup>23</sup>. PPARs are expressed in a variety of body tissues and their location to some extent determines their actions. For example, PPAR $\alpha$  is highly expressed in liver where it plays an important role in regulation of fatty acids metabolism<sup>24</sup>. PPAR $\beta$  is expressed in all body tissues and it is involved in some disorders like cancer, infertility, and dyslipidemia<sup>24</sup>. PPAR $\gamma$  is expressed at high level in adipose tissue where it regulates adipocyte differentiations<sup>25,26</sup>. PPAR $\gamma$ -1 and PPAR $\gamma$ -2 are two isoforms of PPAR $\gamma$ <sup>27</sup>. They differ in the number of amino acid where PPAR $\gamma$ -2 has extra 20 amino acids more than PPAR $\gamma$ -1<sup>28</sup>. Like other PPARs, PPAR $\gamma$  is composed of an N-Terminal Domain (NTD), DNA Binding Domain (DBD) and Ligand Binding Domain (LBD)<sup>29</sup> (Figure 1A ).The ligand binding pocket for PPAR $\gamma$  is larger when compared to other NRs, allowing it to have a variety of ligands<sup>30,31</sup>. The most common intracellular ligands for PPAR $\gamma$  are polyunsaturated fatty acids, such as Linolenic acid and 15-deoxy-delta prostaglandin J2<sup>32</sup>. Thiazolidinedione's (TZDs) anti-diabetic agents are also found to play a role in activating PPAR $\gamma$ .



**Figure 1: (A) Molecular structure of PPAR $\gamma$ . (B) Mode of action of PPAR $\gamma$**

PPAR $\gamma$  form a heterodimer with retinoid X receptor (RXR) that will bind to peroxisome proliferator response element (PPRE) in the targeted gene promoter to regulate gene expression of several proteins<sup>29</sup> (Figure 1B). PPAR $\gamma$  act as transcription factor for several proteins that are involved in lipid metabolism, such proteins are adipocyte fatty acid-binding protein, acyle-CoA synthase, and lipoprotein lipase<sup>33</sup>. Moreover PPAR $\gamma$  represses some genes that are involved in insulin resistance like tumor necrosis factor alpha (TNF- $\alpha$ ), leptin, plasminogen activator inhibitor-1, resistin, interleukin-6 (IL-6), and 11- $\beta$ -hydroxysteroid dehydrogenase type-1<sup>34</sup>.

PPAR $\gamma$  has an important role in lipid storage through induction of adipocyte differentiation and fatty acid flux into adipocytes. PPAR $\gamma$  induces the expression of the phosphoenolpyruvate carboxykinase ( *PEPCK* ) gene which is a characteristic gene of mature adipocyte<sup>35</sup>. Other studies have shown the effect of PPAR $\gamma$  in differentiation of fibroblastic cells into small adipocytes and pre-adipocytes into small adipocytes<sup>36,37</sup>. PPAR $\gamma$  also induce the storage of fatty acid in adipocyte by the activation of lipoprotein lipase that stimulate its release from lipoprotein particles<sup>38</sup>.

### **Brn3b:**

Brn-3b is a member of POU family of transcription factors<sup>39</sup>. POU family of transcription factors consists of common domain of highly conserved regions. These regions consist of POU-specific domain and the POU homeodomain. Brn-3b is one member of Brn3 group that consist of, in addition to Brn-3b, Brn-3a and Brn-3c<sup>40,41</sup>. Several studies have shown

the expression of Brn-3b mRNA in sensory neurons<sup>40,42,43</sup>, reproductive tissues<sup>44</sup> and visual system<sup>45</sup>.

Recent studies by Hindle et al<sup>46</sup> have also shown that Brn-3b is significantly reduced (-1.68 fold) in blood taken from obese patients with T2D when compared with obese non-diabetic subjects. Results of preliminary studies have shown that Brn-3b mRNA can be measured from RNA extracted from peripheral blood and more importantly, that Brn-3b expression is significantly reduced in patients with T2D compared with normal non-diabetic controls.

These preliminary findings obtained from analysis of human blood have identified a link between reduced Brn-3b and obesity/T2D and has led us to hypothesize that:

- Loss of Brn-3b in muscle and adipose tissue are associated with obesity and deregulation of glucose uptake and metabolism.
- Increased Brn-3b expression upon weight loss may be important for restoring normal metabolic processes.

In this study we aim to investigate the expression of PPAR $\gamma$  and Brn-3b in adipose tissue of obese patients who are scheduled for weight reducing sleeve gastroectomy. Moreover we will test the expression of PPAR $\gamma$  and Brn-3b in the different cellular fractions of both subcutaneous and omental adipose tissue. The influence of gender and the presence of metabolic disease (hypertension and diabetes) on the expression of PPAR $\gamma$  and Brn-3b will also be examined.

## **CHAPTER 3: METHODOLOGY**

**Study design:**

A prospective cross-sectional study was conducted on unrelated Arab subjects. Human adipose tissue specimens were collected to investigate if the adipose tissue expresses the transcriptional factor Bnr-b3 and if it has any relation with the metabolic features of these subjects.

Adipose tissue specimens were collected from morbidly obese patients (BMI  $\geq 40$  kg/m<sup>2</sup>) with and without diabetes, dyslipidemia and hypertension (metabolic diseases). All patients (males and females) were scheduled for gastric sleeve surgery with energy restriction to help reduce their body weight. Obese patients were recruited from metabolic surgery unit, Hamad Medical Corporation, from September 2013 to March 2014. All subjects who participated in this study signed a consent form in accordance with the deceleration of Helsinki 2000, after detailed explanation of the study. Two types of abdominal adipose tissue were collected from patients, subcutaneous and omental.

The patients were classified into two groups based upon their insulin sensitivity: insulin resistant and insulin sensitive. . The medical history and medication of the patients were recorded as well as fasting HbA1c (%) and serum concentration of glucose, high-density lipoprotein (HDL)-cholesterol, and total cholesterol, and insulin. Samples were collected during the surgical procedure and immediately transported to the Life Sciences Research Division of the Anti-Doping Laboratory Qatar where it was immediately further processed by collagenase digestion to isolate the adipocytes and the stromal vascular cells, followed by RNA extraction and q-PCR.

**Ethical consideration:**

Ethical approval for the study protocol was obtained from both the Institutional Review Boards of Hamad Medical Corporation and Anti-Doping Laboratory Qatar. Confidentiality of data was assured by the use of anonymized identity numbers on all data recording

forms. The principal investigator holds the link between names and identity numbers in forms placed in secured location that satisfies the local requirements for data protection.

**Sample preparation:**

1g of fresh adipose tissue was used to fractionate adipose tissue to its cellular component using collagenase enzyme (Gibco, cat. #: 17100-017).

100ml of Fresh collagenase solution was prepared by mixing 86.67ml of 2mM CaCl<sub>2</sub>, 13.3ml of 7.5% BSA and 0.5g of collagenase Type1 from Gibco (cat.#: 17100-017).

1ml of collagenase per 1g of tissue was added, the tube placed horizontally in a shaking water-bath and incubated for 1-hour at 37°C. After incubation, the tubes were centrifuged for 5 minutes at 1790 rpm. Then the sample was taken out and shaken vigorously to disrupt the pellet and mix the cells. The centrifugation was repeated at 1790 rpm for 5 minutes. After centrifugation the samples showed several separated layers: Top oil and fat layer, yellow layer of floating adipocyte cells, underlying layer of collagenase solution, and a dark red layer of SVF at the bottom of the tube. The oil and collagenase layers were discarded and the adipocytes (yellow layer of floating cells) were carefully transferred to a new tube and taken for RNA isolation.

10ml of prewarmed PBS with 1%BSA was added to the remaining SVF layer and transferred to a new tube and centrifuged at 1790 rpm for 5 minute. Any remaining collagenase and oil, if present, was removed. 3ml of Erythrocyte lysis buffer was then added and incubated for 10 minutes at room temperature. After incubation, the sample was centrifuged at 1790 rpm for 5 minute and the lysis buffer removed before proceeding to RNA isolation.

**RNA extraction:**

RNA extraction was performed using Trizol reagent (Invitrogen cat. #: 15596-026) as described in the manual.

1ml of TRIzol per 50-100mg of tissue sample was added and pipetted up and down several times to lyse the cells and then incubated for 5 minutes at room temperature. Chloroform (0.2ml chloroform per 1ml Trizol) was added, vortexed vigorously for 15 second and then centrifuged at 14000 rpm for 15 minute at 4 °C. After centrifugation the aqueous phase was collected into a new tube and 0.5ml of 100% Isopropanol added to precipitate the nucleic acids. The sample was incubated at -20 for at least 1 hour or overnight, followed by centrifugation at 14,000 rpm for 15 minutes at 4°C. After centrifugation the isopropanol was removed very carefully leaving the RNA pellet. The RNA pellet was washed twice with 1ml of 75% ethanol per 1ml Trizol and centrifuged at 14,000 rpm for 5 minutes at 4 °C. After the last wash, the ethanol was removed carefully from tube and the pellet allowed to air dry on ice with for 10 minutes. The pellet was resuspended in 30µl of RNase free water and incubated at 56°C for 10 minute. The sample was left on ice for a further 10 minutes before being stored at -80°C.

### **Measuring Quality and Quantity of RNA:**

The RNA was quantified by measuring the A260/A280 ratio using the NanoDrop ND-1000 V3.7.1 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). The Nucleic Acid Bio-analyzer 2100 (Agilent) was used to determine the quality of the RNA.

### **cDNA synthesis:**

cDNA was synthesized by taking 200ng of RNA using High Capacity cDNA Reverse Transcription Kit (life technology, Cat.#4369914). The following reaction volumes were used for cDNA synthesis:

Component	Volume/Reaction	(µL)
10x RT Buffer	2.0	2
25×dNTP Mix (100 mM)		0.8
10x RT Random Primers		2
MultiScribe™ Reverse Transcriptase		1
Nuclease-free H <sub>2</sub> O		4.2
<b>Total per Reaction</b>		<b>10</b>

10  $\mu$ L of 200ng RNA was added to the above reaction mix and reverse transcriptase performed in the thermal cycler. The RT enzyme is activated by incubation at 25°C for 15 minute followed by transcription at 37°C for 2 hours, with a final inactivation step at 85°C for 5 minute. The newly synthesized cDNA was stored at -20 °C for RT-qPCR.

### **RT-qPCR:**

The relative quantitation of gene expression was performed using real-time polymerase chain reaction (RT-PCR). Taqman probes for  $\beta$ -actin (Cat. #: Hs99999903), PPAR $\gamma$  (Cat. #: Hs01115513) and Brn-3b (Cat. #: Hs00960964) genes were ordered from Applied Biosystem with amplicons length of 171bp, 90bp and 51bp respectively.

PCR was performed by adding 10 $\mu$ l TaqMan Gene Expression Master Mix (Applied Biosystems, Cat. #: 436901), 1 $\mu$ l TaqMan probe, 1  $\mu$ l cDNA and 8 $\mu$ l RNase-free water to make total volume of 20 $\mu$ l. Reaction mix was run on the ViiA™ 7 Real-Time PCR System (Applied Biosystems) according to the manufacture protocol.

### **Data analysis:**

Cycle threshold (Ct) is defined as the cycle number at which a significant increase in the fluorescence signal compared to background emission is first detected. Calculating Ct values for each sample was performed to quantify unknown samples.  $\Delta$ Ct was defined for each sample by subtracting the  $\beta$ -actin gene Ct from each selected gene Ct. Relative gene expression was calculated by the  $2^{-\Delta\Delta Ct}$  method, i.e. the negative exponential power of 2 of each sample's  $\Delta$ Ct minus the  $\Delta$ Ct of a reference sample, which kept constant throughout the experiments.

### **Statistical Analysis:**

The results are expressed as mean  $\pm$  SD for all normally distributed data or as median (interquartile ranges) for non-parametric values. Differences among experimental groups were evaluated by Mann-Whitney U test for non-normally distributed data. Spearman



Rank correlation was used to evaluate the relationship between BMI and gene expression of our study subjects as the data was skewed. The figures show Pearson Correlation data as the non-parametric results could not be plotted as a graph to show the direction of the relationship, while the strength of the relationship is shown using Spearman Rank  $r$  values. Significance was defined as two-tailed  $P < 0.05$ . All statistical analyses were performed using the SPSS program for Windows (version 21 statistical software; Texas instruments, IL, USA).

## **CHAPTER 4: RESULTS**

### Patient's Demographic data:

The clinical data and biological parameters of the patients recruited in the study are summarized in Table 1. The clinical data includes medical history of the patients, systolic, diastolic and mean arterial blood pressure (SBP, DBP and MAP), and systemic glucose and insulin levels. All patients were obese with an average age of 42 years old and BMI of 45.9 Kg/m<sup>2</sup>. According to the clinical data, patients were classified into 2 groups: Metabolically Healthy Obese (MHO) and Pathological Obese. MHO were characterized by fasting plasma glucose (FPG) < 7.8 mmol/L, **insulin level < 6.5 miU/mL**, SBP<140 mmHg, DBP<85 mmHg and have no metabolic diseases. The other group, PO, were characterized by having FPG <7.8 mmol/mL, **insulin level > 7.0 miU/mL** with or without any metabolic diseases. Of the 9 patients, 3 patients were classified as MHO therefore they are used as a control in this study.

**Table 1: Clinical data and biological parameter of the patients:**

Sample Number	Gender	History	Age	Weight (kg)	BMI	SBP	DBP	MAP	Glucose	Insulin	MHO/ PO
1	F	No history	34	128	53	110	80	90	6.8	13.75	PO
2	M	Hypertension	40	124	40.5	130	70	90	5.5	16.85	PO
3	F	Diabetes & Hypertension	41	128	39.5	132	70	90.7	4.9	11.31	PO
4	F	No history	51	112	48	168	94	118.7	7.1	11.34	PO
5	M	Hypertension	46	118	40.8	140	70	93.3	4.4	27.21	PO
6	M	Diabetes	17	98	36.4	130	6.8	98	6.9	8	PO
7: Control	F	No history	42	98.5	46.5	120	74	89.3	6.2	4.25	MHO
8: Control	F	No history	39	98	41	130	80	96.7	5.4	4.65	MHO
9: Control	M	No history	29	157	40	130	80	96.7	12.8	5.62	MHO

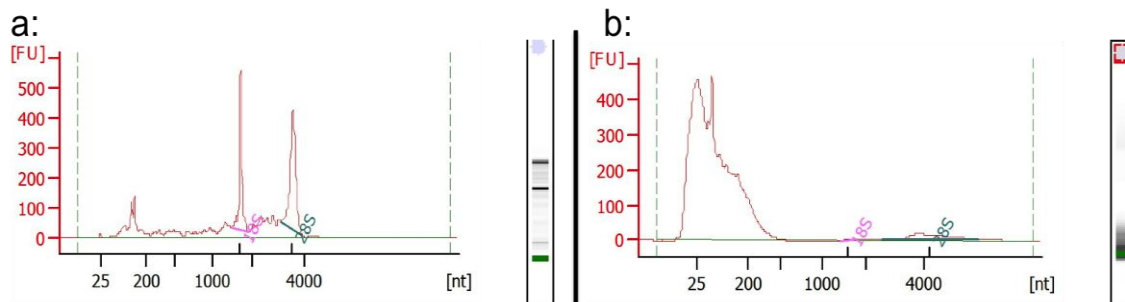
### Adipose Tissue treatment and RNA extraction:

Subcutaneous and omental adipose tissues were collected from patients at the time of the operation. Collagenase digestion separates the adipose tissue to its cellular components: stromal vascular fraction (SVF) and adipocytes (ADIPO). Each patient, therefore, had 4

samples: SC-SVF, SC-ADIPO, OM-SVF and OM-ADIPO. Table 2 shows concentrations of RNA for each sample and Figure 2 shows the results of bio-analyzer for checking the quality of the isolated RNA. RNA with poor quality (Figure 2 -b) was excluded from the analysis as they may interfere with accuracy of the results.

**Table 2: List of RNA concentrations:**

Sample Number	Adipose tissue fraction	Concentrations (ng/ $\mu$ l)	Sample Number	Adipose tissue fraction	Concentrations (ng/ $\mu$ l)
1	SVF-SC	19.7	5	SVF-SC	153.1
	SVF-OM	44		SVF-OM	127
	ADIPO-SC	33.6		ADIPO-SC	73.3
	ADIPO-OM	61.7		ADIPO-OM	141.9
2	SVF-SC	84.3	6	SVF-SC	85.1
	SVF-OM	53.2		SVF-OM	257.6
	ADIPO-SC	220.9		ADIPO-SC	333.7
	ADIPO-OM	163.3		ADIPO-OM	474.6
3	SVF-SC	139.2	7	SVF-SC	91.2
	SVF-OM	210.9		SVF-OM	78.5
	ADIPO-SC	191.6		ADIPO-SC	83.8
	ADIPO-OM	3048.7		ADIPO-OM	43
4	SVF-SC	83.3	8	SVF-SC	95.6
	SVF-OM	66.8		SVF-OM	325.4
	ADIPO-SC	77.2		ADIPO-SC	227.7
	ADIPO-OM	78.5		ADIPO-OM	188.4
Control	SVF-SC	191.3	Abbreviation:		
	SVF-OM	28.2	SVF: Stromal Vascular Fractions, ADIPO:		
	ADIPO-SC	47.5	Adipocyte, OM: Omental, SC: Subcutaneous		
	ADIPO-OM	66.1			



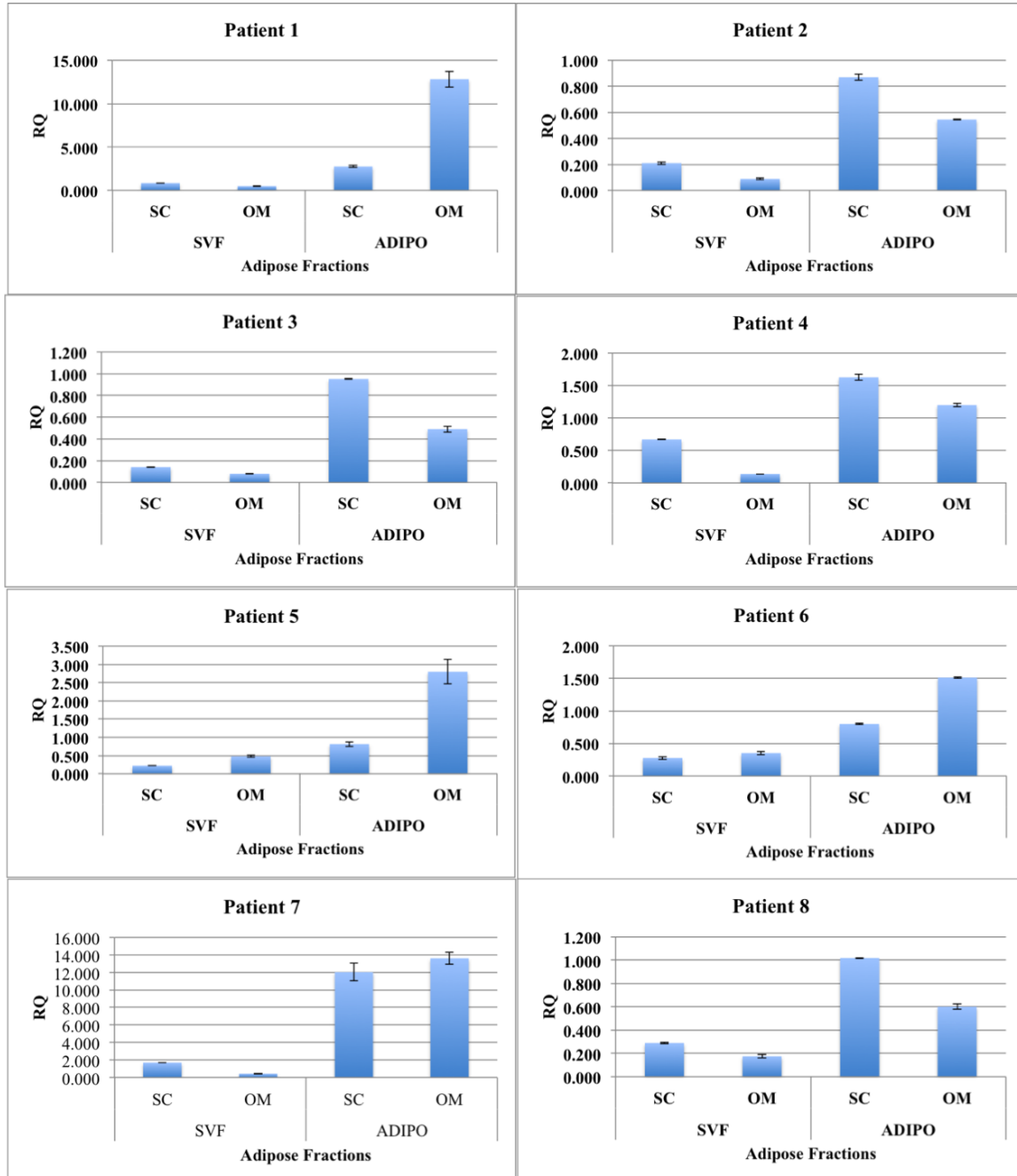
**Figure 2: Comparison between good (a) and poor (b) quality of RNA**

### Gene expression:

#### PPAR $\gamma$ :

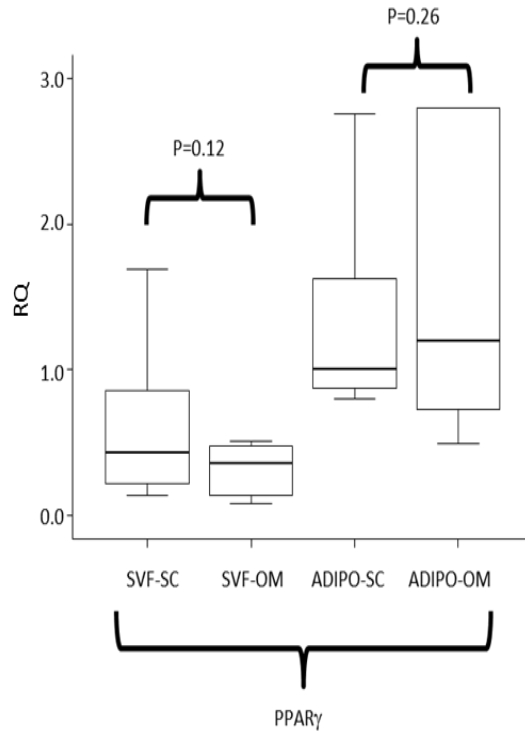
Results of PPAR $\gamma$  expression for individual patients are shown in Figure 3. As can be seen the data are skewed and the median (and interquartile range) of mRNA expression of PPAR $\gamma$  is shown in Figure 4. PPAR $\gamma$  was expressed in both subcutaneous and omental

adipose tissue. Compared to SVF, adipocytes showed significantly higher expression of PPAR $\gamma$  in tissues from both depots (sc: p=0.025; om p=0.05; Figure 5 A and B). Further, unlike in adipocytes, the SVF expression of PPAR $\gamma$  was significantly higher in cells of the subcutaneous adipose tissue than in omental adipose tissue (p=0.042).



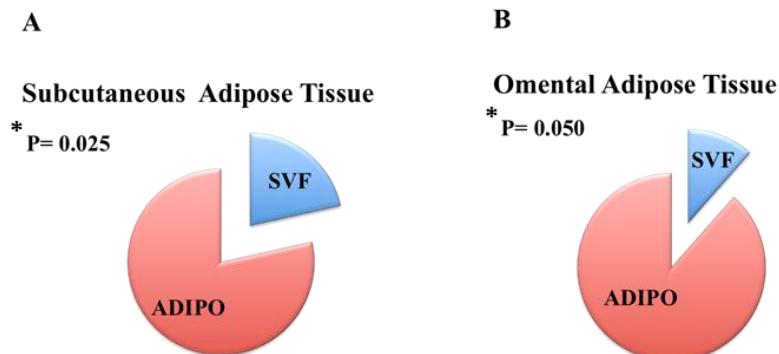
**Figure 3: mRNA expression of PPAR $\gamma$  for each patient.**

Data are shown as mean ( $\pm$  SD). SVF: Stromal Vascular Fractions, ADIPO: Adipocyte, OM: Omental, SC: Subcutaneous



**Figure 4: mRNA expression of PPAR $\gamma$ .**

Data are shown as median ( $\pm$ Interquartile range). SVF: Stromal Vascular Fractions, ADIPO: Adipocyte, OM: Omental, SC: Subcutaneous.



**Figure 5: Comparison of PPAR $\gamma$  expression in (A) subcutaneous and (B) omental adipose tissue.** Data are shown as mean. SVF: Stromal Vascular Fractions, ADIPO: Adipocyte,

**Mediators of PPAR $\gamma$  expression:**

Figure 6 and 7 shows positive Pearson correlations between PPAR $\gamma$  expressions in adipocyte of subcutaneous and omental tissues and BMI of the patients (Spearman Rank correlations: SC:  $r=0.80$ ,  $p=0.01$  & OM:  $r=0.52$ ,  $p=0.15$ ). Thus, as BMI increased so did the expression of PPAR $\gamma$  in adipocytes of the subcutaneous and in the omental depots.

There was also significantly higher expression of PPAR $\gamma$  in the adipocytes of the subcutaneous depot of females compared to males (Figure 8).

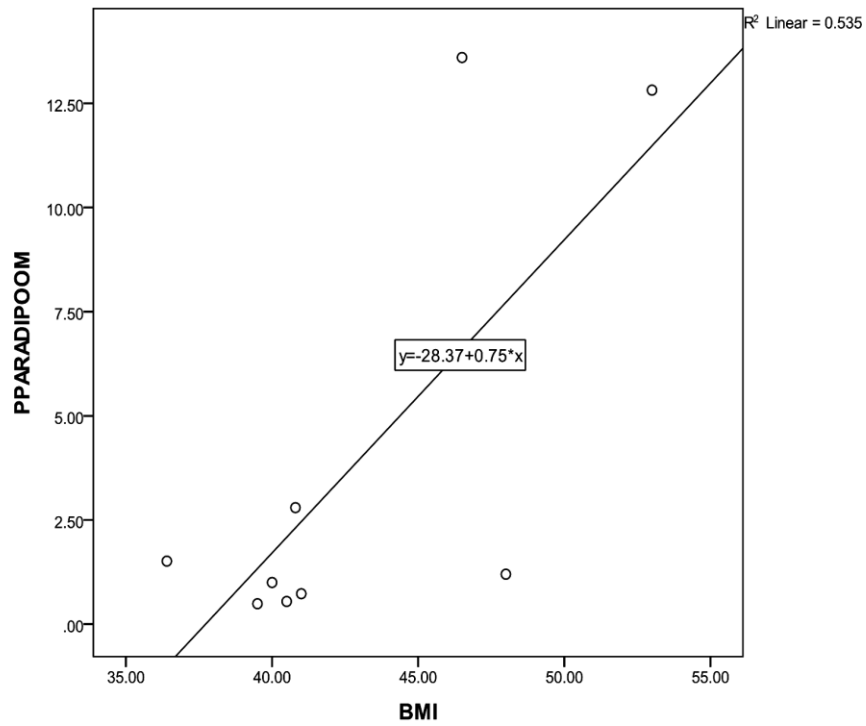


Figure 6: Pearson Correlation between BMI and the expression of PPAR $\gamma$  in adipocytes of omental adipose tissue

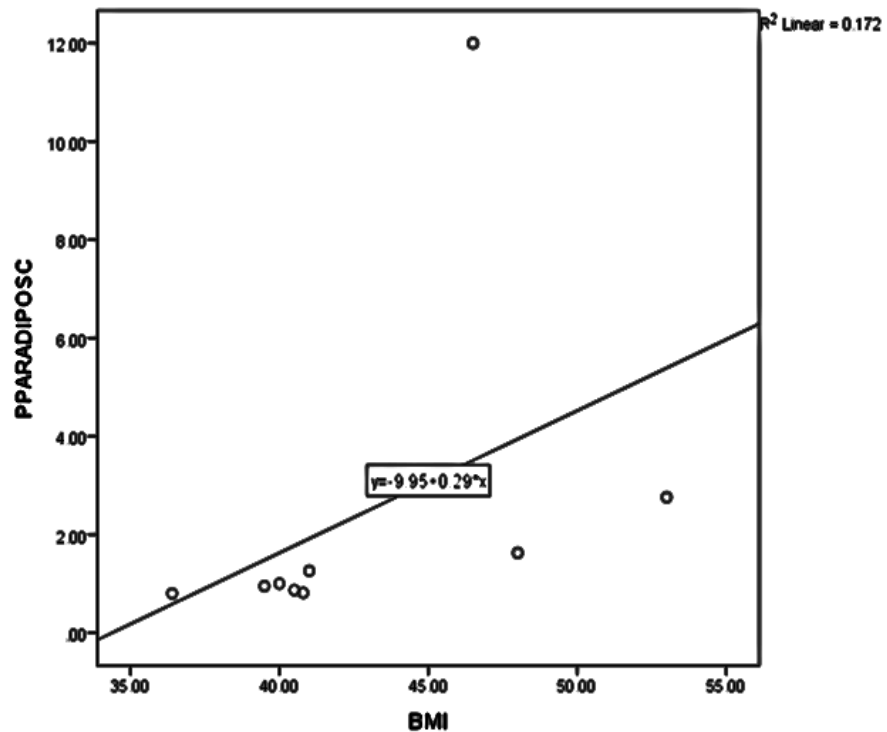
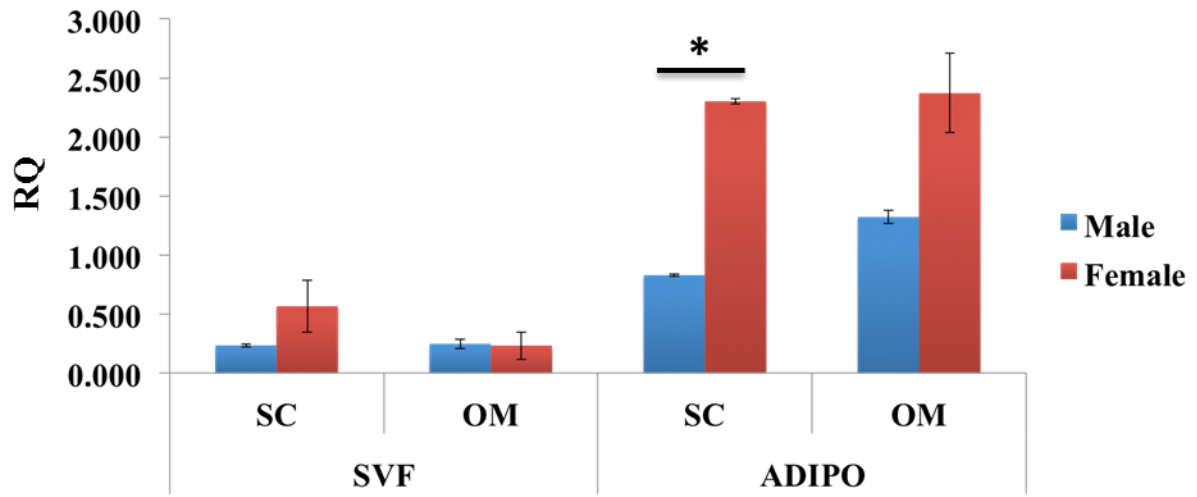


Figure 7: Pearson Correlation between BMI and the expression of PPAR $\gamma$  in adipocytes of subcutaneous adipose tissue.



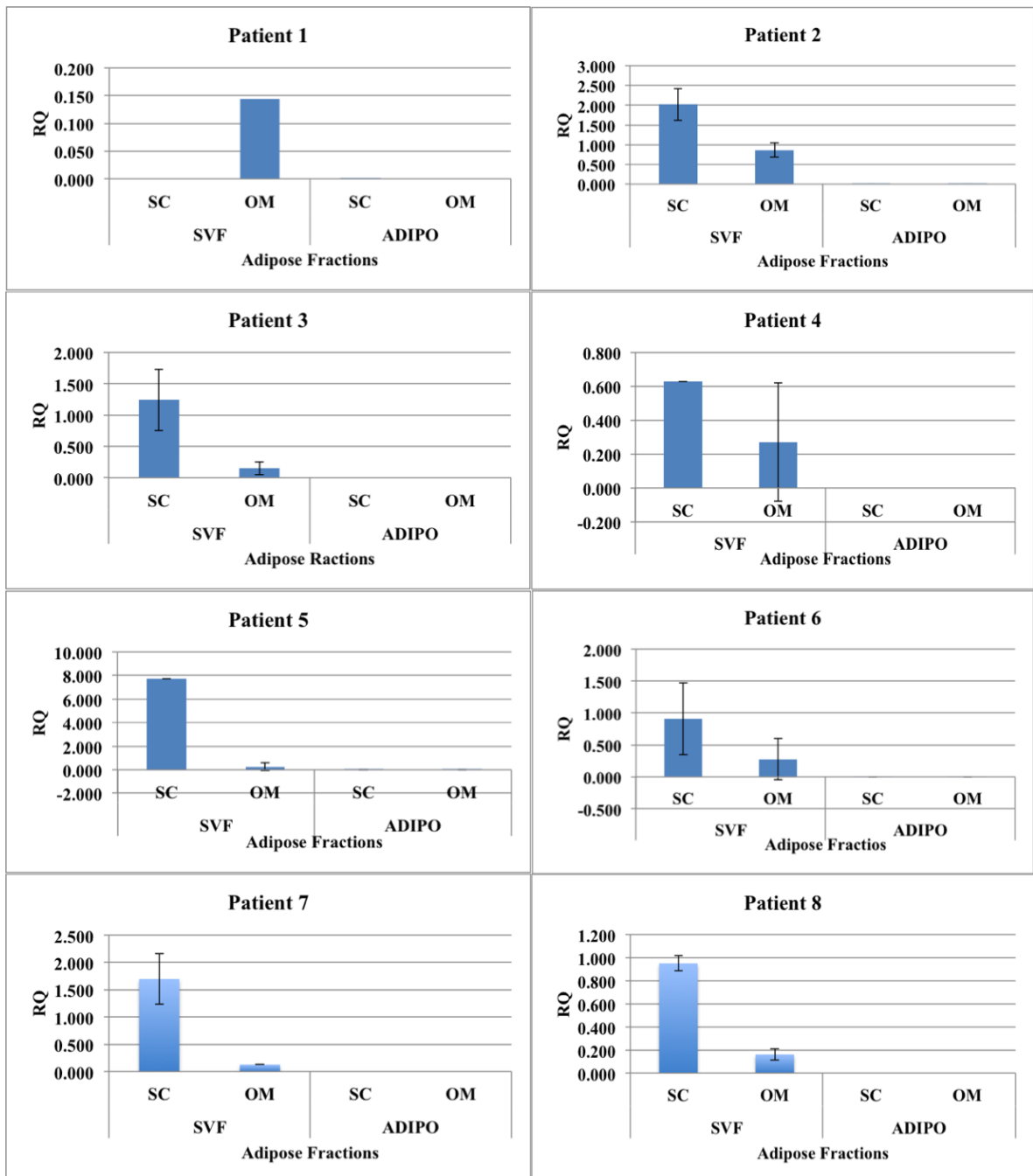
**Figure 8: mRNA expression of PPAR $\gamma$  in male and female.**

Data are shown as mean ( $\pm$  SD). SVF: Stromal Vascular Fractions, ADIPO: Adipocyte, OM: Omental, SC: Subcutaneous. Comparisons are Mann-Whitney U  $p$ -values and significance at  $<0.05$  is shown by \*.

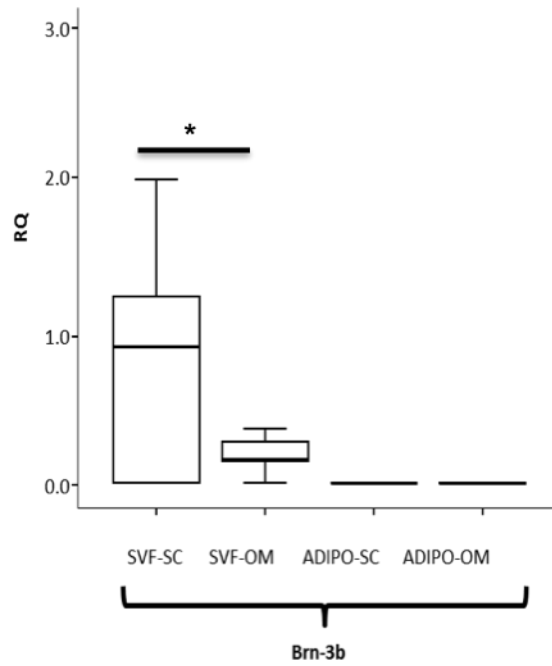


**Brn-3b:**

Expression of Brn-3b of all patients is shown in Figure 9. Figure 10 shows the median (and interquartile range) of mRNA expression of Brn-3b. Brn-3b was expressed in the SVF of both subcutaneous and omental adipose tissue ( $P=0.007$ ), while expression was not detected in the adipocytes of either adipose tissue depot.



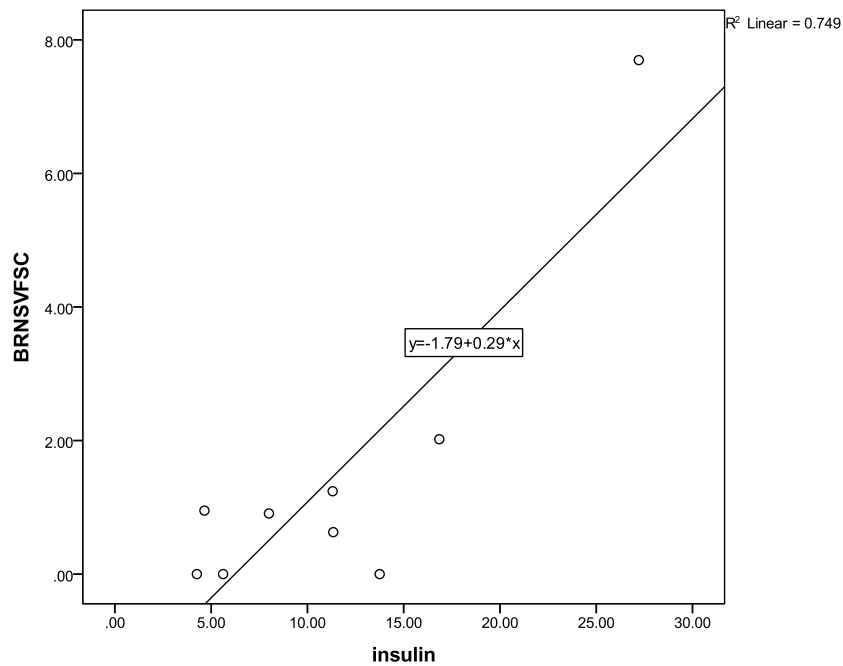
**Figure 9: mRNA expression of Brn-3b for each patient:** Data are shown as mean ( $\pm$  SD). SVF: Stromal Vascular Fractions, ADIPO: Adipocyte, OM: Omental, SC: Subcutaneous.



**Figure 10: mRNA expression of Brn-3b:** Data are shown as median ( $\pm$ Interquartile range). SVF: Stromal Vascular Fractions, ADIPO: Adipocyte, OM: Omental, SC: Subcutaneous. p values significance at  $<0.05$  is shown by \*

### Mediators of Brn-3b:

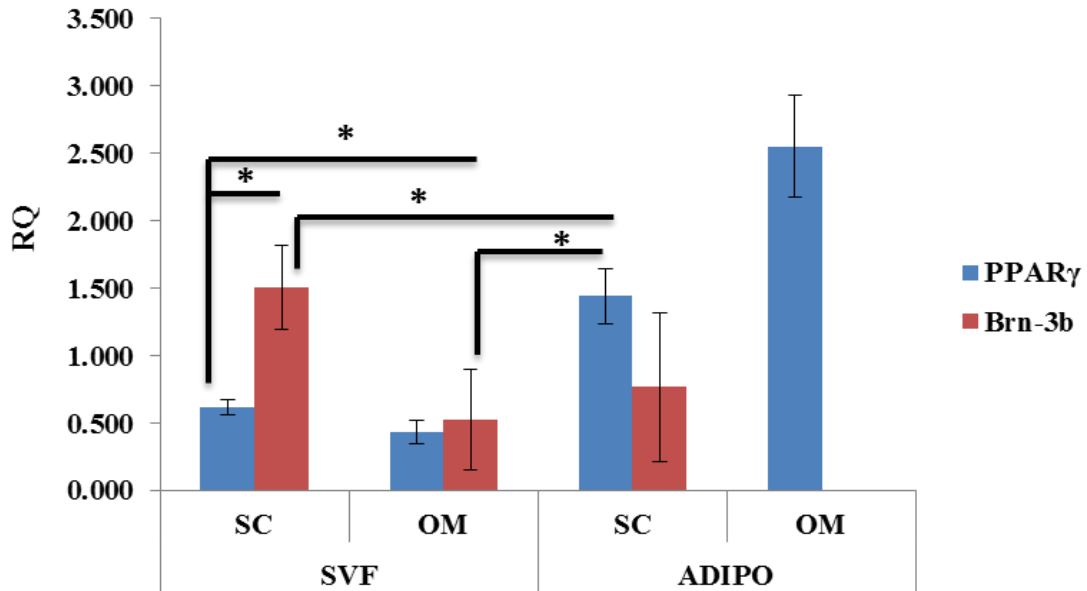
Figure 11 shows positive correlation between insulin level and expression of Brn-3b in the SVF of subcutaneous adipose tissue.



**Figure 11: Correlation between insulin level and the expression of Brn-3b in SVF of subcutaneous adipose tissue.**

**Brn-3b vs. PPAR $\gamma$ :**

Interestingly there were negative associations between the expression of Brn-3b and PPAR $\gamma$  (Figure12). Table 3 shows Pearson rank correlation and Figure 13 shows Pearson correlations between both transcription factors.

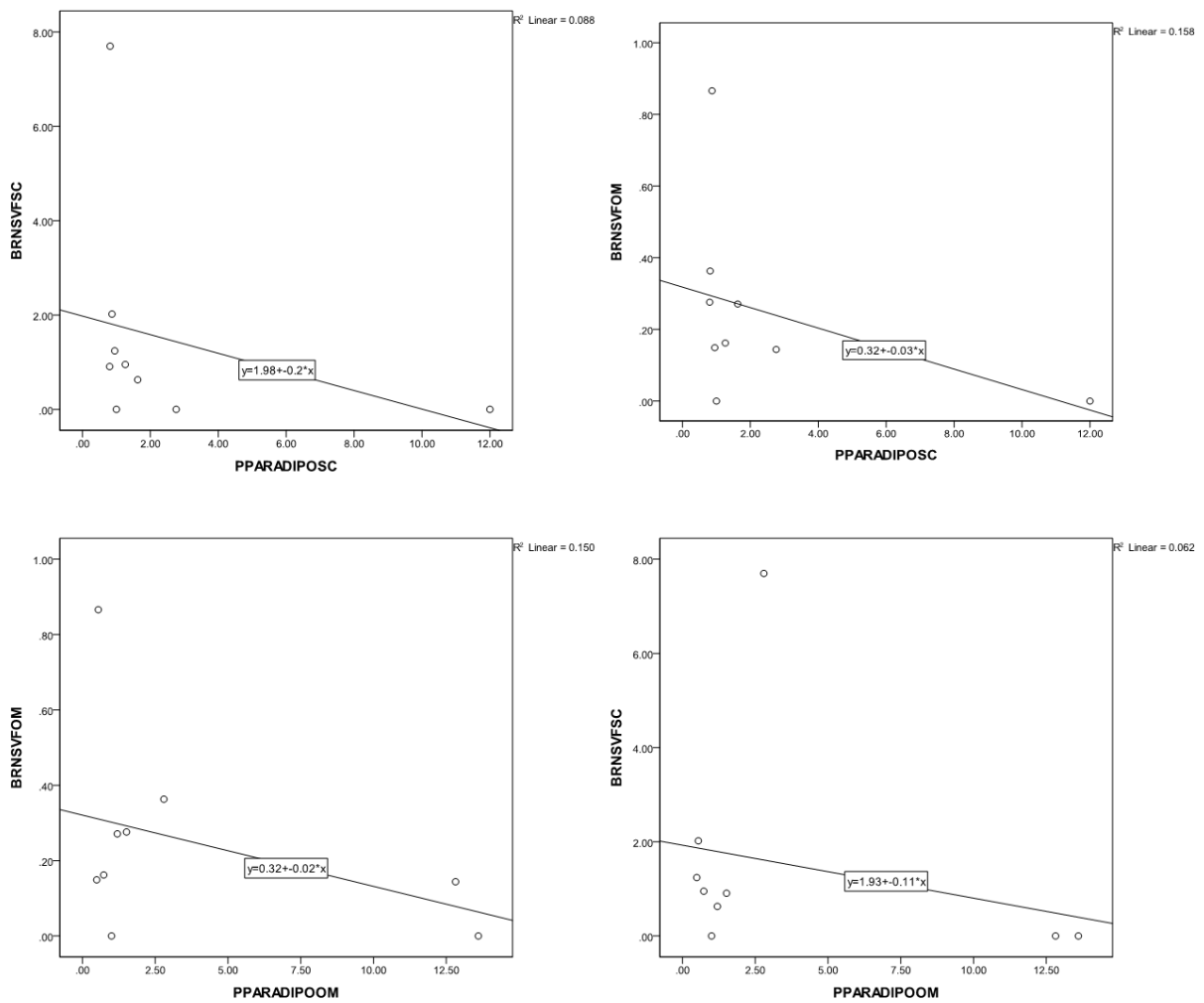


**Figure 12: mRNA expression of PPAR $\gamma$  and Brn-3b in subcutaneous and omental adipose tissue.**

Data are shown as mean ( $\pm$  SD). SVF: Stromal Vascular Fractions, ADIPO: Adipocyte, OM: Omental, SC: Subcutaneous. Comparisons are Mann-Whitney U and p values, significance at  $<0.05$  is shown by \*

**Table 3: Spearman Rank correlation between PPAR $\gamma$  and Brn-3b expression in subcutaneous and omental adipose tissue.**

	Brn-3b-SVF-SC	Brn-3b-SVF-OM
PPAR $\gamma$ -SVF-SC	R= - 0.898	R= - 0.728
	P= 0.001	P= 0.026
PPAR $\gamma$ -ADIP-SC	R= - 0.712	R= - 0.703
	P= 0.031	P= 0.035



**Figure 13: Pearson correlations between PPAR $\gamma$  and Brn-3b expression in subcutaneous and omental adipose tissue**

**PPAR $\gamma$ , Brn-3b and metabolic disease:**

PPAR $\gamma$  expression was significantly elevated in both the adipocytes ( $p=0.016$ ) and in the SVF ( $p=0.016$ ) of the sub-cutaneous depots of the patients without any history of metabolic disease (no diabetes or hypertension).

Conversely, Brn-3b expression was elevated in the SVF of both the subcutaneous ( $p=0.032$ ) and the omental ( $p=0.063$ ) tissue of the patients with metabolic disease, compared to those without.

## **CHAPTER 5: DISCUSSION**

The global increase in the rate of obesity and its associated comorbidities has led researchers to try and understand the molecular mechanisms that underlie the relationship between obesity and disease. Several transcription factors are known to play a role in obesity. Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) is an example of one of these transcription factors. This transcription factor plays a major role in the regulation of both adipocyte differentiation and metabolism. Furthermore, synthetic ligands of PPAR $\gamma$ , the thiazolidinedione family of compounds are already used as therapeutic agents in diabetes and obesity.

An unpublished study of obese patients showed that expression of another transcription factor, Brn-3b, was significantly reduced in whole blood of diabetic patients. The role of this factor in obesity and diabetes, as well as in the adipose tissue, is all, as yet, unexplored.

The aim of this study was, firstly, to ascertain the expression of PPAR $\gamma$  and Brn-3b in subcutaneous and omental adipose tissue of obese patient undergoing weight-reducing sleeve gastroectomy. Furthermore, the expression of PPAR $\gamma$  and Brn-3b was also determined in the different cellular fractions, adipocytes and the SVF, of both subcutaneous and omental adipose tissue. Lastly, interactions between the expression of the two transcription factors themselves, as well as with other mediators, such as gender, body mass and the presence of metabolic diseases were investigated.

We found that PPAR $\gamma$  is expressed in the adipocytes of both subcutaneous and omental adipose tissue. This result is consistent with the literature where it has been shown that PPAR $\gamma$  is highly expressed in adipose tissue and is a key regulator of differentiation of preadipocytes to mature adipocyte.<sup>25,26</sup> The stromal vascular fractions (SVF) showed lower expression of PPAR $\gamma$  compared to that in adipocytes. The SVF consists of preadipocytes, fibroblasts, vascular cells, neuronal cells, and immune cells such as adipose

tissue macrophages. The low expression of PPAR $\gamma$  in SVF probably reflects lower expression in preadipocytes compared to adipocytes, as only on the onset of adipogenesis is there an increase in PPAR $\gamma$  which is responsible for the events leading to the cellular growth arrest prior to differentiation into adipocytes, as well as the induction of adipocyte specific genes<sup>47</sup>. As these preadipocytes within the SVF are derived from adult humans, especially those with morbid obesity, it may also reflect their lower capacity to expand this adipose tissue depot. Another possible explanation is that this expression of PPAR $\gamma$  is due to adipocytes contaminating the SVF fraction. This is one limitation of the study that could have been controlled for by screening for adipocyte markers, such as adiponectin or adipisin. In keeping with data showing that females have more subcutaneous adipose tissue than males<sup>48</sup>, we found that PPAR $\gamma$  expression in adipocytes of subcutaneous adipose tissue of females was higher than that of males (p=0.027). Our data also showed a positive correlation between BMI and PPAR $\gamma$  expression in the adipocytes of the subcutaneous adipose tissue. Both these confirm findings for PPAR $\gamma$  expression in cells of the adipose tissue from previous studies and act as an ample control for our findings with Brn-3b.

This study is the first that has investigated the expression of Brn-3b in human adipose tissue. We found that Brn-3b is significantly expressed in SVF cells of subcutaneous adipose tissue compared to omental adipose tissue. There was little or no expression of Brn-3b in adipocytes, either because it is not expressed in adipocytes or it is shut down during adipogenesis. We found a significant correlation between Brn-3b and insulin levels, where the expression of Brn-3b in SVF especially of the subcutaneous adipose tissue is increased with the rising systemic insulin level. This suggests that a hyperinsulinaemic state is likely to be marked by elevated expression of Brn-3b. In keeping with this we found that while PPAR $\gamma$  expression was significantly elevated in both the adipocytes and in the SVF of the sub-cutaneous depots of the patients without any history of metabolic

disease (no diabetes or hypertension), Brn-3b expression was elevated in the SVF of both the subcutaneous and the omental tissue of the patients with metabolic disease, compared to those without. Patients with metabolic disease are often hyperinsulinaemic. We also found significant inverse relationships between adipocyte PPAR $\gamma$  and SVF Brn-3b of both adipose tissue depots. This relationship needs further investigation, and it also raises questions concerning the implication and the role of Brn-3b in the pathology of obesity and T2DM.

In other data from our group it was found that murine preadipocytes and adipocytes do not express Brn-3b. If this is confirmed in humans, and this current data points to that, then other cells within the SVF, such as smooth muscle cells, endothelial cells and/or neuronal cells are likely to be the major cells that express this transcription factor. The depot specific difference seen here would then reside within changes in these cells depending on the depot from which they derive.

Obesity and T2DM pose a serious threat to human health, but the molecular mechanisms that predispose to this disease are not understood. Our data supporting the role for Brn-3b in obesity and hyperinsulinaemia, both linked with increased risk for T2DM may have implications for human health and must be investigated further. Whether we can readily measure Brn-3b in peripheral blood is yet to be ascertained. If this were to confirm our results in the SVF this would be a significant advantage because blood samples are more readily accessible for analysis than biopsies from metabolically active tissues such as muscle, adipose tissue or liver. By measuring Brn-3b levels in pre-and post-surgery blood samples from a larger number of patients, we could assess how altered expression correlates with weight loss/BMI as well as clinical parameters such as weight, fasting plasma glucose, insulin and HOMA-IR (index of insulin resistance). We could also determine whether such levels are affected by age.



Furthermore, there is a Brn-3b knockout mouse model that is available and will be useful in determining whether intake of high fat can affect the expression of Brn-3b and how this correlates with weight gain and metabolic dysfunction. This model can also be helpful to test if weight-loss is associated with reversal of Brn-3b expression which is important if loss of Brn-3b turns out to be important for weight gain and development of T2DM. The Brn-3b KO mutant studies will provide a good model to determine how loss of Brn-3b affect gene expression changes linked to weight gain and metabolic dysfunction. Furthermore this model will be invaluable for identifying potential Brn-3b target genes in metabolically active tissue so that we can determine how this regulator controls normal glucose uptake/metabolism.

In conclusion our study indicated that there was a reciprocal relationship between the expression of PPAR $\gamma$  and Brn-3b that needs further investigation, and raises questions concerning the implication of Brn-3b in the pathology of obesity and T2DM.

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